Expression and localization of vascular endothelial growth factor A (VEGFA) and its two receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine corpus luteum and utero-placental compartments during pregnancy and at normal and induced parturition

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Abstract

VEGFA is one of the most potent known inducers of angiogenesis. However, the function of angiogenic factors in the canine corpus luteum (CL) of pregnancy and in the pregnant uterus and placenta has not yet been elucidated. Therefore, here we investigated the expression and localization of VEGFA and its receptors (VEGFR1/FLT1 and VEGFR2/FLK1/KDR) in the canine CL and utero-placental compartments (ut-pl) throughout pregnancy until prepartum luteolysis. Antigestagen-mediated effects on expression of VEGF system in ut-pl were elucidated in mid-pregnant dogs. While displaying high individual variation, the luteal VEGFA was elevated during pre-implantation and post-implantation, followed by a decrease during mid-gestation, which was more pronounced at the mRNA level, and showed constant expression afterwards. Within the uterus, it increased following implantation and during mid-gestation in ut-pl compartments, but was downregulated at prepartum luteolysis. Luteal VEGFR1 expression resembled that of VEGFA; VEGFR2 remained unaffected throughout pregnancy. In ut-pl compartments, both receptors increased gradually towards mid-gestation; a prepartum decrease was observed for VEGFR1. Antigestagen-treatment resulted in decreased expression of ut-pl VEGFR1. In the CL, VEGFA stained in luteal cells. Uterine signals of VEGFA and its two receptors were observed in epithelial and vascular compartments, and in myometrium. In placental labyrinth, additionally, trophoblast stained positively. Luteal VEGFR1 was localized to the luteal cells and tunica media of blood vessels, whereas VEGFR2 stained only in capillary endothelial cells. The upregulation of luteal and the ut-pl VEGF system during early gestational stages supports the increased vascularization rate during this time. The diminishing effects of the prepartum endocrine milieu on VEGFA function seem to be more pronounced in the ut-pl units.

Key words: domestic dog, corpus luteum (CL), uterus-placenta complex (ut-pl), angiogenesis, vascular endothelial factor (VEGF) system

1 Introduction

In the reproductive tract, angiogenesis is an essential biological process involved in decidualization, implantation and placentation, as well as corpus luteum (CL) formation. It is regulated by the orchestration of diverse factors such as prostaglandins (PGs) and growth factors (Fraser and Wulff, 2003; Reynolds et al., 2000; Sherer and Abulafia, 2001). Among these, vascular endothelial growth factor (VEGF) is one of the most potent angiogenic agents (Ferrara and Davis-Smyth, 1997). By acting through its two tyrosine kinase receptors, VEGFR1 (FLT1) and VEGFR2 (FLK1/KDR), VEGF modulates migration and proliferation of endothelial cells and also induces vascular permeability (Cebe-Suarez et al., 2006; Stuttfeld and Ballmer-Hofer, 2009).

The luteal expression and/or tissue concentration of VEGF is tightly correlated with development of the CL as shown, *e.g.*, in primates, including humans, and cattle (Berisha et al., 2000; Ravindranath et al., 1992; Yamamoto et al., 1997). Its highest levels in growing CL correspond to the high rate of proliferation and vascularization. As clearly shown in rats and mice, interfering with the biological activity of VEGF at the level of its receptors, either VEGFR1 or VEGFR2, results in impaired formation and function of the CL by preventing angiogenesis (Ferrara, 1998; Zimmermann et al., 2001). Interestingly, however, no effects on preexisting vasculature were seen, indicating the importance of the VEGF system primarily for neo-angiogenesis (Beck and D'Amore, 1997). Furthermore, by interacting with angiopoietins, *e.g.*, Ang1, VEGF supports integrity of blood vessels and maintains their stability (Hanahan, 1997; Hazzard and Stouffer, 2000). Being itself a luteotropic factor, VEGF responds positively to LH in luteinizing granulosa cells and the developing CL, and its expression is also triggered by hypoxia (Christenson and Stouffer, 1997; Nishimura, 2010).

Less information is available concerning the expression and function of the VEGF system in the canine CL. Most of the knowledge relates to non-pregnant cyclic animals. Thus, VEGFA expression is increased during the early luteal phase, and decreases by mid-diestrus (Mariani et al., 2006). The decreased proliferative activity observed during the second half of diestrus is associated with reduced density of the vascular network, as concluded from the lower expression of endothelial cell-bound expression of endoglin (Hoffmann et al., 2004a; Papa and Hoffmann, 2011). The decreased vascularization rate does not change significantly afterwards, until the end of the luteal life span (Hoffmann et al., 2004a), indicating that the diminished blood supply is not the major factor responsible for the slowly progressing luteal regression in non-pregnant dogs (Hoffmann et al., 2004b). In accordance with this, only recently unaltered levels of luteal transcripts encoding for the VEGF system were shown during normal and induced prepartum luteolysis/abortion (Kowalewski, 2014). The initially increased expression of vasculogenic factors is accompanied by elevated luteal expression of transcripts encoding for hypoxia-inducible factor 1α (HIF1α) and fibroblast growth factor 2 (FGF2) in the early CL from non-pregnant bitches (Papa et al., 2014).

In the uterus, proper endometrial angiogenesis and vascular permeability are important for the successful onset of pregnancy (Sherer and Abulafia, 2001). VEGF is involved in these processes by coordinating the biochemical and functional changes associated with estrogen-dependent uterine edema, vascular permeability and remodeling (Cullinan-Bove and Koos, 1993; Rockwell et al., 2002; Shifren et al., 1996). High levels of uterine VEGF during early peri-implantation stages of pregnancy were found in several species, *e.g.*, rabbit, hamster, rat, mouse and human (Das et al., 1997; Douglas et al., 2009; Rabbani and Rogers, 2001; Sugino et al., 2002; Yi et al., 1999). Similarly, in the dog, increased uterine levels of transcripts encoding for VEGFA and its

receptors were found in response to free-floating, pre-implantation embryos (Bukowska et al., 2011; Schafer-Somi et al., 2013). The localization and spatio-temporal expression of these factors within the canine uterus and placenta, however, have not yet been established. Blockage of VEGF in rats resulted in markedly decreased endometrial estrogen-induced microvascular permeability (Rockwell et al., 2002). Following implantation, an inadequate vascular supply may result in preeclampsia, fetal growth restriction or death (Barut et al., 2010; Cerdeira and Karumanchi, 2012; Jarvenpaa et al., 2007), and VEGF is among the most important factors involved in regulating placental angiogenesis (Reynolds and Redmer, 2001). Accordingly, in mice, knockout of either of the VEGF receptors, *i.e.*, VEGFR1 and/or VEGFR2, results in multiple vascular malformations and consequently in embryonic lethality (Fong et al., 1995; Shalaby et al., 1995). Across species, *e.g*, in humans, sheep and pigs, the placental localization of VEGF is targeted to fetal trophoblast cells, showing a pregnancy stage-dependent expression pattern (Bogic et al., 2001; Demir et al., 2004; Kimberly, 2001; Winther et al., 1999).

Taking into consideration the essential role of the VEGF system during the establishment and maintenance of pregnancy, this study for the first time thoroughly describes the expression and localization patterns of VEGFA, VEGFR1 and VEGFR2 in canine CL of pregnancy, as well as in utero-placental compartments collected from dogs at selected stages throughout pregnancy. Additionally, in order to investigate possible functional interplay between the VEGF system and progesterone receptor (PGR) signaling at the utero-placental level, the expression of target genes was assessed during antigestagen-induced abortion in mid-pregnant dogs.

2 Materials and Methods

2.1 Tissue collection and preservation

Tissue material utilized for the present study was collected by ovariohysterectomy (OHE) from healthy, mixed breed dogs at selected time points during pregnancy. The day of mating was designated as day 0 of gestation for all bitches and was 2-3 days after ovulation. The time of ovulation was detected by measuring plasma progesterone levels (>5 ng/ml in peripheral blood) at 2-3 day intervals and by vaginal cytology. The tissue samples were classified as: pre-implantation (days 8-12, n=5), post-implantation (days 18-25, n=5), mid-gestation (days 35-40, n=5) and prepartum luteolysis (n=3). The pre-implantation stage of pregnancy was detected by uterine flushing and recovery of pre-implantation, free-floating embryos under a stereomicroscope. According to the descriptions by Amoroso (Amoroso, 1952) and Kehrer (Kehrer, 1973), during the post-implantation stage of canine pregnancy the utero-placental compartments are being formed, whereas at mid-gestation fully developed utero-placental compartments are observed. The prepartum luteolysis was ascertained by regular measurements of circulating P4 at 6h intervals from day 58 of gestation onwards, until a marked decrease of P4 was observed in three consecutive measurements, eventually reaching levels below 3ng/ml, when OHE was performed. In addition, abortions were induced by administering Aglepristone (Alizine®, Virbac, Bad Oldesloe, Germany; 10mg/Kg bw, 2x/24 h apart) to mid-pregnant bitches (days 40-45 of pregnancy, n=10). Tissue samples were collected either 24h (n=5) or 72h (n=5) after the second treatment. All experimental procedures were performed according to the guidelines of animal welfare legislation and approved by the respective authorities of Justus-Liebig University in Giessen, Germany (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c-

20/15c GI 18,14), and the ethics committee University of Ankara (permit no. Ankara 2006/06), Ankara, Turkey.

The tissue samples collected comprised: Corpora lutea (CL) and uterine samples (from the pre-implantation stage of pregnancy), or utero-placental compartments (ut-pl), representing the full thickness, middle part of the girdle, avoiding the marginal hematoma when derived from later stages of pregnancy following implantation and/or following Aglepristone treatment. Immediately after sampling and removal of excess connective tissue, samples were washed with phosphate-buffered saline (PBS) and processed as described before (Gram et al., 2013; Kowalewski et al., 2010; Kowalewski et al., 2011). Briefly: for immunohistochemistry (IHC), tissues were fixed with 10% neutral PBS-buffered formalin for 24h at +4^{o}C, followed by washing in PBS for one week and embedded in paraffin wax; for total RNA isolation, samples were incubated in RNAlater® (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24h at +4°C and then stored at -80°C until analysis.

2.2 Immunohistochemistry

Immunohistochemical staining was performed using our previously described standard immunoperoxidase method (Kowalewski et al., 2006a; Kowalewski et al., 2006b). Briefly, formalin-fixed paraffin-embedded tissue sections (2-3µm thick) were placed on Super Frost® microscope slides (Menzel-Glaeser, Braunschweig, Germany); xylene was used for deparaffinization, then a graded ethanol series was applied for rehydration. After subsequent washing under tap water, epitope retrieval was performed in a microwave oven at 560 W for 15 min in 10mM citrate buffer pH 6.0. For VEGFA and VEGFR1 localization in luteal samples, enzymatic digestion was performed with 0.25% pepsin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 10mM HCl for 10 min at 37°C, instead of heat-induced antigen retrieval.

Endogenous peroxidase activity blocking was done by treating slides with 0.3% hydrogen peroxide in methanol. In order to reduce background staining, sections were incubated with either 10% normal goat serum or 10% normal horse serum, depending on the source species of the secondary antibody. They were then incubated overnight at +4°C with mouse monoclonal anti-VEGF (ab68334; for ut-pl tissue sections diluted 1:100, for luteal samples 1:300) purchased from Abcam, Cambridge, UK. Rabbit affinity-purified polyclonal anti-VEGFR1/Flt-1 (sc-316; for ut-pl tissue sections diluted 1:300, for luteal samples 1:350) was purchased from Santa Cruz Biotechnology Inc., CA, USA, and rabbit monoclonal anti-VEGFR2/KDR/Flk-1 (2479S; for ut-pl tissue sections the dilution was 1:50, for luteal samples 1:300) from Cell Signaling Technology Inc., Danvers, MA, USA. Negative controls consisted of samples omitting the primary antibody, and non-immunized IgG of the same species instead of primary antibody used at the same dilution and protein concentration as the primary antibody (the so-called isotype control).

This was followed by incubation with biotin-labeled secondary antibody incubation at a 1:100 dilution. The following secondary antibodies were used for 30 min: biotinylated goat anti-rabbit IgG BA-1000 and horse anti-mouse IgG BA-2000 (both from Vector Laboratories Inc., Burlingame, CA, USA). For amplification of signals, the streptavidin-peroxidase Vectastain ABC kit (Vector Laboratories, Inc.) was added to sections for 30 min, and finally they were treated with the Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, Switzerland) to visualize peroxide activity. Slides were counterstained with hematoxylin, dehydrated in a graded ethanol series, equilibrated in xylene, and mounted in Histokit (Assistant, Osterode, Germany).

2.3 Total RNA isolation and Real Time (TaqMan) PCR

Total RNA was extracted from tissue samples with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Concentrations of RNA were examined with

188 a NanoDrop 2000C® spectrophotometer (Thermo Fisher Scientific AG, Reinach, Switzerland).
189 Afterwards, samples were DNAse-treated with RQ1 RNase-free DNase (Promega, Dübendorf,
190 Switzerland) and reverse transcribed (RT reaction) following our previously published protocol
191 and utilizing random hexamers as primers and other reagents from Applied Biosystems by
192 Thermo Fisher Scientific, Foster City, CA, USA (Kowalewski et al., 2011; Kowalewski et al.,
193 2006b). Semi-quantitative real time (TaqMan) PCR reaction was performed in an automated
194 fluorometer (ABI PRISM 7500 Sequence Detection System from Applied Biosystems by Thermo
195 Fisher Scientific). The primers and probes used in the PCR reaction were designed using Primer
196 Express software version 2.0 (Applied Biosystems by Thermo Fisher Scientific) and were
197 purchased from Microsynth, Balgach, Switzerland. Selected amplicons of each gene were sent for
198 commercial sequencing (Microsynth). The list of primers, 6-carboxyfluorescein (6-FAM) and 6-
199 carboxytetramethylrhodamine (TAMRA)-labelled TaqMan probes, and the length of PCR
200 products are presented in Table 1. Autoclaved water and the so-called RT-minus controls
201 (samples that were not reverse-transcribed) served as negative controls. Each reaction mixture
202 comprised: 200nM TaqMan Probe, 300 nM of each primer, 12.5 µl Fast Start Universal Probe
203 Master (ROX)® (Roche Diagnostics, Mannheim, Germany) and 5µl cDNA corresponding to
204 100ng total RNA per sample. The amplification conditions were as follows: denaturation at 95°C
205 for 10 min, 40 cycles at 95°C for 15 sec and 1 cycle at 60°C for 60 sec. The efficiency of each
206 target gene was calculated using the CT slope method, ensuring approximately 100% reaction
207 efficiency, and quantification was performed using a comparative CT method (ΔΔCT method)
208 according to the manufacturer's protocols for the ABI PRISM® 7500 Sequence Detection
209 System (Applied Biosystems) and as described previously (Kowalewski et al., 2011; Kowalewski
210 et al., 2006b). The sample with the lowest concentration of the target gene served as the

calibrator in the ΔΔCT method. Three reference genes were used for normalizing the results: GAPDH, 18SrRNA (Table 1), and cyclophilin. A commercially available gene-specific TaqMan® system of canine-specific cyclophilin A was purchased from Applied Biosystems by Thermo Fisher Scientific (Prod. No. Cf03986523_gH).

Due to the uneven distribution of data, logarithmic transformation was performed for normalization of the data and the geometric means (Xg) ± deviation factors were calculated for the analysis of target gene expression. The effects of the observational group on target gene mRNA levels were assessed by applying a parametric one-way analysis of variance (ANOVA). In cases of $P<0.05$, multiple comparisons tests were applied: the Tukey-Kramer multiple comparisons test in experiments showing temporal changes in gene expression during the course of pregnancy, and Dunnett's multiple comparisons test in experiments showing the expression of target genes after Aglepristone-induced luteolysis. In the latter experiments, the results present the n-fold change in gene expression compared to its mRNA-levels in ut-pl collected at mid-gestation. All tests were performed using the statistical software program GraphPad 3.06 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Time-dependent expression of the VEGF-system in canine CL and ut-pl compartments throughout pregnancy.

The expression of mRNA encoding for VEGFA and its two receptors (VEGFR1 and VEGFR2) was detectable in all tissue samples investigated. While the luteal VEGFA and VEGFR1 were significantly modulated over time ($P=0.01$ and $P<0.001$, respectively) (Fig. 1A,B), the expression of VEGFR2 did not change significantly ($P=0.31$) (Fig. 1C). VEGFA was elevated at the

beginning of the luteal phase with highest values noted post-implantation. This was followed by a significant decrease at mid-gestation ($P<0.05$) with no further changes later on, during prepartum luteolysis (Fig. 1A). A similar expression pattern was observed for luteal VEGFR1 mRNA levels, which were strongly increased post-implantation and significantly lower afterwards, at mid-term and during prepartum luteolysis ($P<0.001$) (Fig. 1B).

The expression of all three target genes, *i.e.*, VEGFA, VEGFR1 and VEGFR2, in ut-pl compartments was time-dependent ($P=0.002$, $P=0.005$ and $P=0.001$, respectively) (Fig. 2A-C). Implantation and placentation were associated with gradually increasing expression of these target genes towards mid-gestation, showing, however, large individual variations among groups and individuals. This increase was strongest for VEGFR2 mRNA, which was already significantly unpregulated in ut-pl after implantation ($P<0.01$), and further markedly elevated at mid-gestation ($P<0.001$) when compared with pre-implantation (Fig. 2C). The prepartum luteolysis was characterized by significantly decreasing levels of VEGF and VEGFR1 ($P<0.001$ and $P<0.01$, respectively) (Fig. 2A,B), while VEGFR2 levels remained unaffected (Fig. 2C).

3.2 The effect of antigestagen treatment on expression of the VEGF system in ut-pl compartments.

The effect of applying the antigestagen Aglepristone® on expression of the VEGF system in ut-pl compartments was assessed in mid-pregnant dogs. Non-treated mid-pregnant dogs served as controls. Although displaying high individual variation, the expression of VEGFA and VEGFR2 did not change significantly ($P=0.75$ and $P=0.32$, respectively) (Fig. 2D,F), while VEGFR1 mRNA levels were significantly affected ($P=0.006$) and had already decreased 24h after the second treatment ($P<0.01$), remaining suppressed at 72h ($P<0.01$ compared with controls) (Fig. 2E).

3.3 Immunohistochemical detection of the VEGF system throughout pregnancy

IHC was applied for detection of VEGFA, VEGFR1 and VEGR2 expression in canine CL and ut-pl compartments during pregnancy. In CL, VEGFA was localized predominantly in luteal cells, with only weak staining in non-steroidogenic luteal compartments of the CL. While characterized by high individual variations, the signals appeared stronger at post-implantation and mid-gestation stages of pregnancy. Much weaker staining was observed at pre-implantation stage, and only weak or no signals could be detected during prepartum luteolysis (Fig. 3A-D). VEGFR1 expression was localized to the luteal cells and tunica media of blood vessels throughout pregnancy (Fig 4A-D). At the time of prepartum luteolysis, luteal cells seemed to stain more weakly, while signals in the tunica media of vessels were still clearly visible (Fig 4D). As for VEGFR2, it was localized in luteal blood vessels with distinctly stronger signals in endothelial cells of capillaries and weaker staining in the tunica media of some larger vessels (Fig 5A-D). The staining did not vary widely throughout pregnancy. Luteal cells showed weaker or no immunoreactions for VEGFR2 from pre-implantation until prepartum luteolysis.

Prior to implantation, uterine VEGFA signals were localized to the endometrial luminal surface epithelial cells, as well as in superficial and deep uterine glands and myometrium (Fig 6A,B). Deep uterine glands stained more weakly than superficial ones (Fig. 6B). Weaker stromal signals were localized predominantly in vascular endothelial cells (Fig. 6A,B). Following implantation, a similar uterine cellular localization pattern was observed for VEGFA signals in the uterine part of the ut-pl compartments (Fig 6C,D and 6F,G). However, endometrial and myometrial signals appeared to be weaker during prepartum luteolysis (Fig 6F,G). In the placental labyrinth, VEGFA signal intensities increased towards mid-gestation, resulting in strong staining in fetal trophoblast cells (predominantly cytotrophoblast), and in vascular endothelial cells both in the placenta

materna and fetalis; in contrast, only weak or no signals were observed in maternal stroma-derived decidual cells (Fig 6E and 6H). Signal intensities were weaker during prepartum luteolysis (Fig 6H).

The uterine expression of VEGFR1 was localized in superficial and deep uterine glands and myometrium prior to implantation (Fig 7A,B). No or negligible immunostaining was seen in luminal epithelial cells of the endometrial surface (Fig 7A). As for VEGF, some stromal signals were visible in the vascular bed. Implantation was associated with strong staining, especially in the placental parts of the ut-pl units (Fig 7C,D). The pattern of uterine VEGFR1 expression remained constant with, however, only moderate signal intensity (Fig 7C,D and Fig 7F,G). Within the placental labyrinth, strong signals were seen in vascular endothelial cells and in cytotrophoblast cells, whereas only weak signals were localized in syncytiotrophoblast cells and maternal decidual cells (Fig 7E). During prepartum luteolysis, signals appeared weaker with, however, a localization pattern similar to that during earlier stages of pregnancy (Fig 7H). With regard to VEGFR2, its expression in the early pregnant pre-implantation uterus matched the localization pattern of VEGFA with signals localized in luminal surface epithelial cells and superficial and deep uterine glands. Stronger immunoreactions were observed in capillary pericytes, vascular endothelial cells and myometrium (Fig 8A,B). Similar endometrial and myometrial signals were found following implantation and placentation (Fig. 8C,D and 8F,G); the superficial glandular epithelial cells (part of the so-called glandular chambers), myometrium and vascular endothelial cells stained positively. Placental localization of VEGFR2 was detected in fetal trophoblast cells, which appeared to be predominantly cytotrophoblasts, and in vascular endothelial cells of the placenta materna and fetalis (Fig 8E). At prepartum luteolysis, the maternal endothelial cells especially stained strongly for VEGFR2. Only weak staining was noticed for VEGFR2 in maternal decidual cells (Fig 8E and 8H).

306

307 **4. Discussion**

308 The establishment and maintenance of pregnancy strongly depend on the appropriate functioning
309 of luteal, uterine and placental vasculogenic and angiogenic activities, and the VEGF system is a
310 set of master regulators controlling these activities. In the present study, we investigated its
311 spatio-temporal expression in the dog at selected time points during pregnancy, *i.e.*, pre-
312 implantation, post-implantation, mid-gestation, and during prepartum luteolysis.

313 The early luteal phase was characterized by elevated expression of the VEGF system in canine
314 pregnant CL, consistent with similar findings described in other species, *e.g.*, humans, cattle, pigs
315 and sheep (Berisha et al., 2000; Kamat et al., 1995; Redmer et al., 1996; Yamamoto et al., 1997).
316 The expression of transcripts encoding for VEGFA and its VEGFR1 receptor decreased by the
317 mid-luteal phase and did not change until prepartum luteolysis. These findings are in accordance
318 with the expression pattern of the VEGF system observed in the CL of non-pregnant cyclic dogs
319 until late luteal regression (Mariani et al., 2006). Although individual variations were wide, the
320 profiles of the respective protein expression of both factors, i.e., VEGFA and VEGFR1, seemed
321 less dramatic, and showed a strong depression first appearing at prepartum luteolysis. This
322 indicates a possible divergence between the mechanisms regulating their mRNA expression and
323 the protein turnover rates during the regression of canine CL. Taking into account the similar P4
324 profiles observed in dogs regardless of their reproductive status, *i.e.*, in pregnant and non-
325 pregnant bitches, and the postulated initial transient period of gonadotropic independency, this
326 further emphasizes the involvement of inherent regulatory mechanisms controlling canine CL
327 function.

328 Thus, as expected, VEGFA appears to be an essential angiogenic factor during canine CL
329 formation. Furthermore, a functional interplay between the different cellular components in the

canine CL is strongly implied from the distribution pattern of the VEGF system within the CL. Thus, whereas in our study, VEGFA was expressed virtually exclusively in the luteal cells, its receptor VEGFR1 was localized at both locations, *i.e*., in steroidogenic cells and in luteal blood vessels. Moreover, VEGFR2 was co-localized with VEGFR1 in the luteal vascular components. This, indeed, strongly suggests both autocrine and paracrine functions of VEGFA and its receptors within an autoregulatory feedback loop in the canine CL. As for the possible regulatory factors modulating VEGFA expression, the hypoxia-sensitive HIF1α might be involved, as its expression is clearly detectable during the developmental period of canine CL (Papa et al., 2013). Moreover, PGE2 was shown to be a positive regulator of VEGF expression in swine luteal cells *in vitro* (Kowalczyk et al., 2008). Similarly, in rodents, blocking cyclooxygenase 2 (COX2/PTGS2) in the newly formed CL, using the selective COX2 blocker NS-398, decreased not only the steroidogenic luteal capacity and, thereby, serum levels of P4, but also the density of luteal capillary vessels (Sakurai et al., 2005). In line with this, PGE2 is also an important regulator of CL function in dogs during its early development (Kowalewski et al., 2013; Kowalewski et al., 2015). This was clearly shown recently by applying the selective COX2 blocker firocoxib to non-pregnant dogs during early diestrus, which resulted in strong suppression of intraluteal PGE2 synthesis and decreased expression of steroidogenic acute regulatory (STAR) protein (Kowalewski et al., 2015). The possible interplay between these two functional systems, *i.e*., PGs and VEGF system, in regulating STAR expression and luteal P4 output remains to be elucidated in the dog. This is particularly interesting since the steroidogenic potential of VEGF has been implicated also in the cow, where antibody-mediated blocking of VEGF function resulted in suppressed luteal STAR mRNA expression and significantly reduced steroidogenic output (Yamashita et al., 2008).

As for the ut-pl compartments, the expression of VEGFA and its receptors increased following implantation, appearing to be associated with placental development, as indicated by the strong expression of all factors in the fetal parts of the placenta.

At the pre-implantation stage, VEGFA was localized in capillaries and epithelial uterine components, implying a role of VEGFA in establishing the uterine edema and vascular permeabilization needed for embryo attachment and implantation as described in other species (Ancelin et al., 2002; Halder et al., 2000; Rockwell et al., 2002). Accordingly, studies conducted in human and rat uterus showed that VEGF expression is induced by P4 and estrogens (Ancelin et al., 2002; Cullinan-Bove and Koos, 1993; Hyder et al., 2000). This might be also true for dogs, in which uterine P4- and estrogen- receptors (PGR, ERα and ERβ) were colocalized with VEGFA expression during the pre-implantation period (Kautz et al., 2014). Additional studies in rodents established the interrelationship between VEGF activity and estrogen-induced uterine decidualization and implantation (Rockwell et al., 2002). Moreover, VEGFR2-mediated pathways were shown to be involved in regulation of cell differentiation during establishment of pregnancy in mice (Douglas et al., 2009). Also in the dog, as presented herein, VEGFR2 expression was stronger than VEGFR1 in uterine and placental vascular beds, suggesting the important role of this receptor in local VEGFA-dependent angiogenic effects.

Following placentation, endometrial signals for VEGF and its receptors were similar to those observed in the pre-implantation uterus. However, strong placental signals were localized in fetal trophoblast cells, suggesting that the VEGF system might play an autocrine and/or paracrine role in placental angiogenesis, and in maintaining changes in utero-placental vascular permeability, and thereby, in fetal and placental development. Our findings concerning the distribution pattern of VEGF system in trophoblast cells are in agreement with similar studies performed in other

species, *e.g.*, human, sheep or rat (Ahmed et al., 1995; Bogic et al., 2001; Demir et al., 2004; Watanabe, 1998). Whereas during prepartum luteolysis the expression of VEGFR2 remained unaffected, the VEGFA and VEGFR1 were markedly suppressed. In the dog, interfering with P4 function by blocking its binding to PGR triggers the luteolytic cascade, resulting in the prepartum PGF2α increase (Kowalewski et al., 2010). Considering the placenta, this increase must originate in trophoblast cells where COX2 is localized (Kowalewski et al., 2010). Recently, the potential of canine placental and uterine homogenates to convert PGE2 to PGF2α was shown (Gram et al., 2014). In addition, the PGE2-generating enzyme, PTGES, was localized in the fetal trophoblast and responded positively to the suppression of PGR function (Gram et al., 2014; Kowalewski et al., 2010). In the present study, both VEGFA and VEGFR2 exhibited large individual variations upon treatment with the antigestagen Aglepristone®, contrasting with the strongly suppressed expression of VEGFR1. The latter in particular can be seen as a sign of a functional change within the trophoblast towards prepartum PG synthesis and induction of the luteolytic cascade. Suppression of the VEGF system function during both normal and induced parturition/abortion might be involved in facilitating placental detachment.

In conclusion, the VEGF system is actively modulated in canine CL and ut-pl compartments throughout gestation. Whereas, at least at the mRNA level, its expression is strongly diminished in mature, mid-term CL exhibiting a fully developed vascular bed, the strong placental expression of VEGFA and both receptors suggests their involvement not only in placental angiogenesis and vasculogenesis but also during maintenance of placental function, possibly by supporting trophoblast differentiation and/or migration.

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599

Figure legends

Figure 1: Expression of VEGFA, VEGFR1/FLT1 and VEGFR2/FLK1/KDR as determined by Real Time (TaqMan) PCR (Xg ± DF) in canine CL at selected stages of pregnancy (A-C). Bars with different letters in (A) differ at differ at $P<0.05$, and in (B) differ at $P<0.001$.

Figure 2: Expression of VEGFA, VEGFR1/FLT1 and VEGFR2/FLK1/KDR as determined by Real Time (TaqMan) PCR (Xg ± DF) in canine pre-implantation pregnant uterus and in utero-placental compartments (ut-pl) at selected time points of pregnancy (A-C) and during antigestagen (Aglepristone®)-induced luteolysis/abortion (D-F; compared with the mid-gestation group used as non-treated control). Bars with different letters differ: in (A) at $P<0.001$, in (B) and (E) at $P<0.01$. For (C) differences are indicated underneath the graph.

Figure 3: Immunohistochemical (IHC) localization of VEGFA in the canine CL at selected time points of pregnancy, *i.e.*, pre-implantation (A), post-implantation (B), mid-gestation (C) and at prepartum luteolysis (D). VEGFA expression is localized to the lutein cells (solid arrows in A-D). There is no background staining in the isotype control (insert to D).

Figure 4: Immunohistochemical (IHC) localization of VEGFR1/FLT1 in the canine pregnant CL at selected time points of pregnancy, *i.e.*, pre-implantation (A), post-implantation (B), mid-gestation (C) and prepartum luteolysis (D). VEGFR1 expression is localized to the luteal cells (open arrows in A-D) and media of the blood vessels (solid arrows in A-D). There is no background staining in the isotype control (insert to D).

623 **Figure 5:** Immunohistochemical (IHC) localization of VEGFR2/FLK1/KDR in the canine
624 pregnant CL at selected time points of pregnancy, *i.e.*, pre-implantation (A), post-implantation
625 (B), mid-gestation (C) and prepartum luteolysis (D). VEGFR2 expression is predominantly
626 localized to the capillary endothelial cells (open arrows in A-D), while weaker staining is
627 observed in media of some larger vessels (solid arrow in C). Luteal cells stain only weakly or
628 negatively (solid arrowheads in A-D). There is no background staining in the isotype control
629 (insert to D).
630
631 **Figure 6:** Immunohistochemical (IHC) localization of VEGFA in the canine pregnant uterus at
632 selected time points during pregnancy: (A,B) pre-implantation stage, (C-E) utero-placental
633 compartments (ut-pl) at mid-gestation, and in ut-pl at prepartum luteolysis (F-H). (A,B) Pre-
634 implantation, VEGFA is localized to the endometrial luminal (surface) epithelial cells (solid
635 arrows in A), glandular epithelial cells of the superficial and deep uterine glands (open arrows in
636 A and B), myometrium (open arrowheads in B) and capillary pericytes (solid arrowheads in A
637 and B). (C-E) Following placentation (representative pictures taken during mid-gestation) within
638 the ut-pl compartments, endometrial VEGFA expression is localized in the epithelial cells of the
639 superficial endometrial glands (the so-called glandular chambers) (open arrows in C) and deep
640 uterine glands (open arrows in D), media of the vessels (solid arrowhead in C) and myometrium
641 (open arrowheads in D). At prepartum luteolysis (F-G), positive uterine signals are localized in
642 the superficial (open arrows in F) and deep uterine glands (open arrows in G), vascular
643 endothelial cells (solid arrowheads in G) and myometrium (open arrowheads in G). Within the
644 placental labyrinth, signals are localized in fetal trophoblast cells (cytotrophoblast, solid
645 arrowheads in E and H) and maternal endothelial cells (open arrows in E and H) with distinctly
646 stronger signals observed at earlier stages of pregnancy (presented at mid-gestation, E) than

during prepartum luteolysis (H). Maternal stroma-derived decidual cells (open arrowheads in H) display only weak staining. There is no background staining in the isotype control (insert to H).

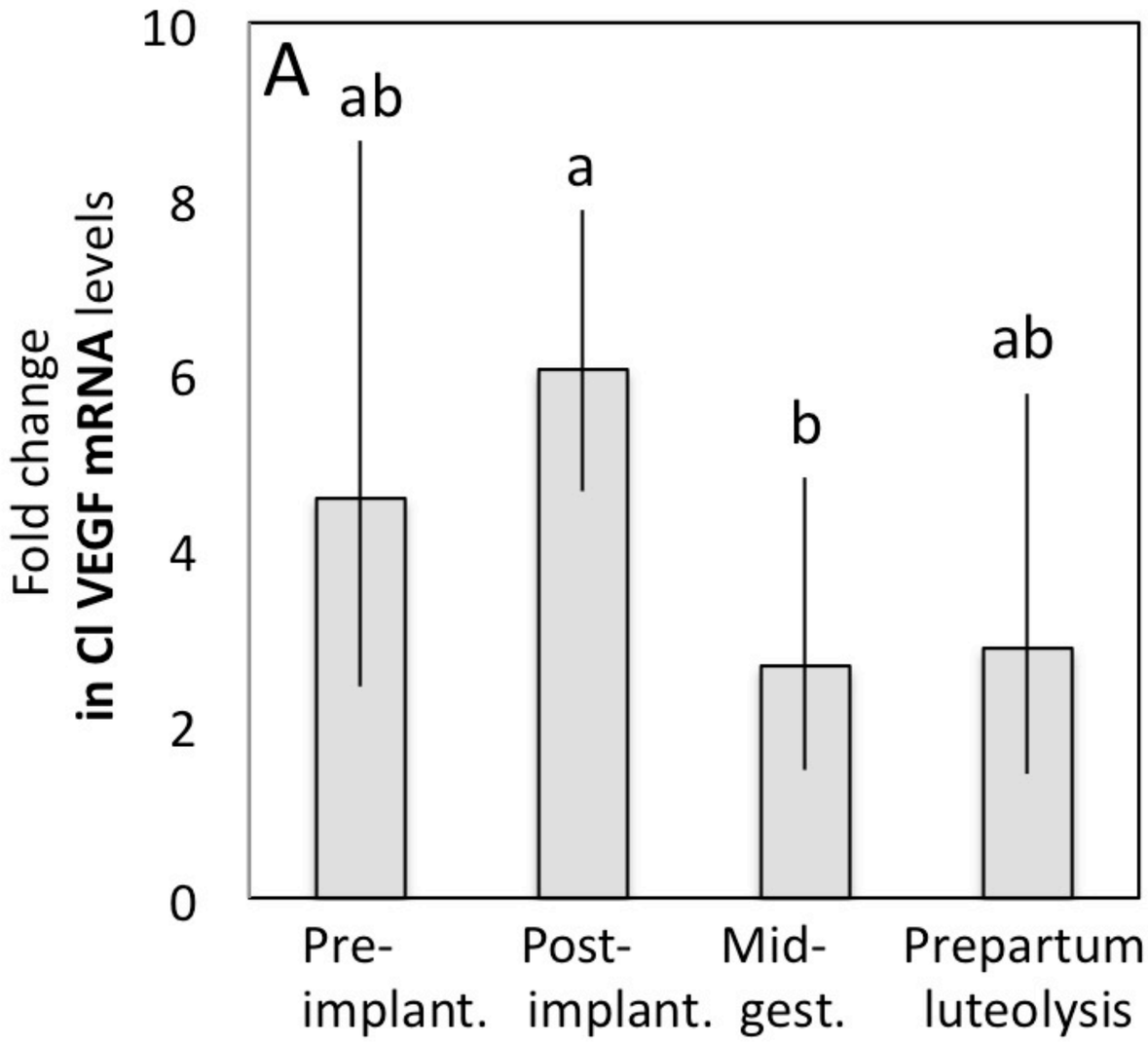
Figure 7: Immunohistochemical (IHC) localization of VEGFR1/FLT1 in the canine pregnant uterus at selected time points during pregnancy: (A,B) pre-implantation stage, (C-E) utero-placental compartments (ut-pl) at mid-gestation, and in ut-pl at prepartum luteolysis (F-H). (A,B) pre-implantation endometrial luminal (surface) epithelial cells stain weakly for VEGFR1 (solid arrows in A). Stronger endometrial signals are localized to the glandular epithelial cells of the superficial and deep uterine glands (open arrows in A and B), and media of the vessels (solid arrowheads in B). Myometrium stains positively (open arrowheads in B). Following implantation (presented at mid-gestation in C and D), and at prepartum-luteolysis (F, G), similar endometrial and myometrial localization patterns are observed. Signals are shown in glandular epithelial cells of the superficial glands (the so-called glandular chambers; open arrows in C and F), deep uterine glands (open arrows in D and G), and myocytes (open arrowheads in D and G). In the placental labyrinth during mid-gestation and prepartum luteolysis, cytotrophoblast cells (solid arrowheads in E and H) are stained, with distinctly stronger signals at earlier stages of pregnancy (represented at mid-gestation in E). Maternal endothelial cells are indicated with open arrows in E and H. Maternal stroma-derived decidual cells display only weak staining (open arrowheads in E and H). There is no background staining in the isotype control (insert to H).

Figure 8 Immunohistochemical (IHC) localization of VEGFR2/FLK1/KDR in the canine pregnant uterus at selected time points during pregnancy: (A,B) pre-implantation stage, (C-E) utero-placental compartments (ut-pl) at mid-gestation, and in ut-pl at prepartum luteolysis (F-H). (A,B) Pre-implantation endometrial luminal (surface) epithelial cells (solid arrows in A),

671 capillary pericytes (solid arrowheads in A and B) and myometrial myocytes (open arrowheads in
672 B) are stained strongly for VEGFR2. Weaker endometrial signals are localized to the glandular
673 epithelial cells of the superficial and deep uterine glands (open arrows in A and B). Following
674 implantation (presented at mid-gestation; C-E), strong endometrial signals are localized in the
675 epithelium of superficial glands (the so-called glandular chambers, open arrows in C), capillary
676 pericytes (solid arrowheads in C and D) and vascular endothelial cells (solid arrow in D). Deep
677 uterine glands (open arrows in D) are stained weakly for VEGFR2. In myometrium, signals are
678 localized in myocytes (open arrowheads in D). At prepartum luteolysis, similar endometrial and
679 myometrial localization patterns are observed (F and G). Signals are localized to the glandular
680 epithelial cells of glandular chambers (open arrows in F) and deep uterine glands (open arrows in
681 G), capillary pericytes (solid arrowheads in F) and myocytes (open arrowheads in G). In the
682 placental labyrinth (presented at mid-gestation) trophoblast cells (cytotrophoblast, solid
683 arrowheads in E), capillary pericytes (solid arrows in E), and maternal endothelial cells (open
684 arrow in E), are predominantly stained. During prepartum luteolysis, strong staining is localized
685 in maternal endothelial cells (open arrow in H). Positively stained cytotrophoblast is indicated
686 with solid arrowheads in H. Only weak staining is observed in maternal decidual cells throughout
687 gestation (open arrowheads in H) and syncytiotrophoblast (solid arrows in H). There is no
688 background staining in the isotype control (insert to H).
689

690 **Table1**

691 List of primers and TaqMan Probes used for the semi-quantitative RT-PCR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Fold change
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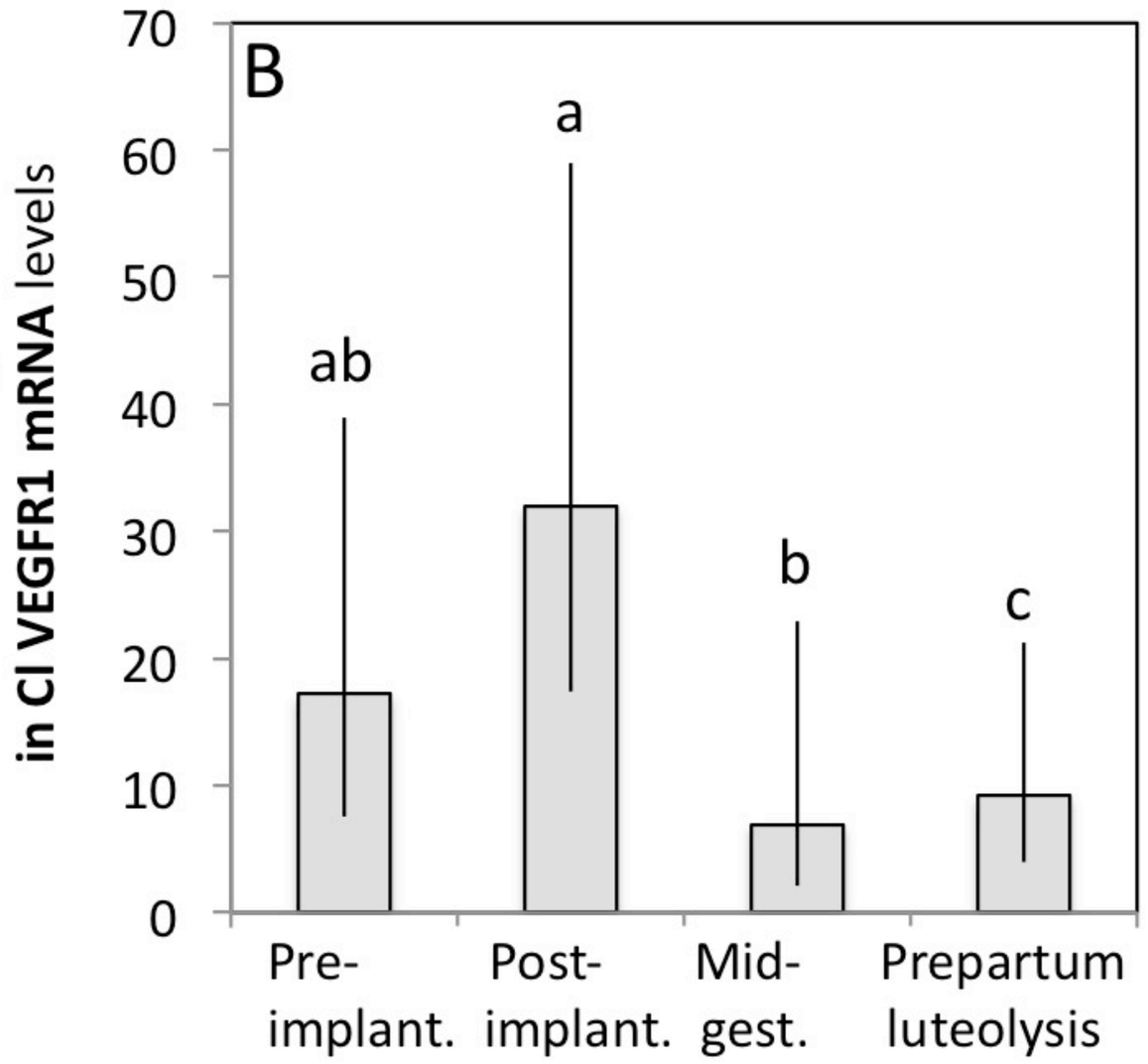
Pre-implant.
Post-implant.
Mid-gest.
Prepartum luteolysis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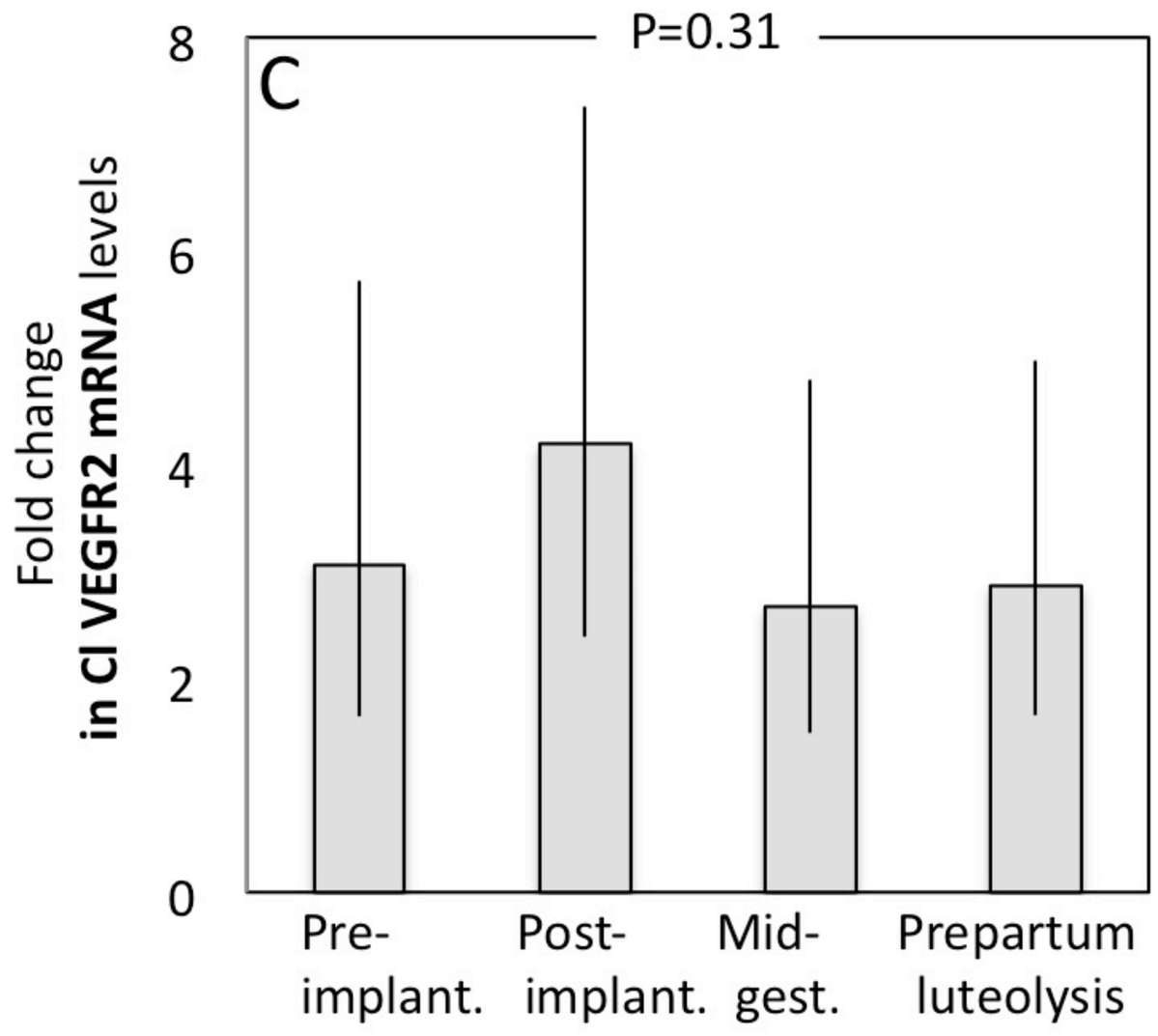
Post-implant.
Mid-gest.
Prepartum luteolysis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Fold change
in CI VEGFR2 mRNA levels
Pre-implant.
Post-implant.
Mid-gest.
Prepartum luteolysis

Figure 1

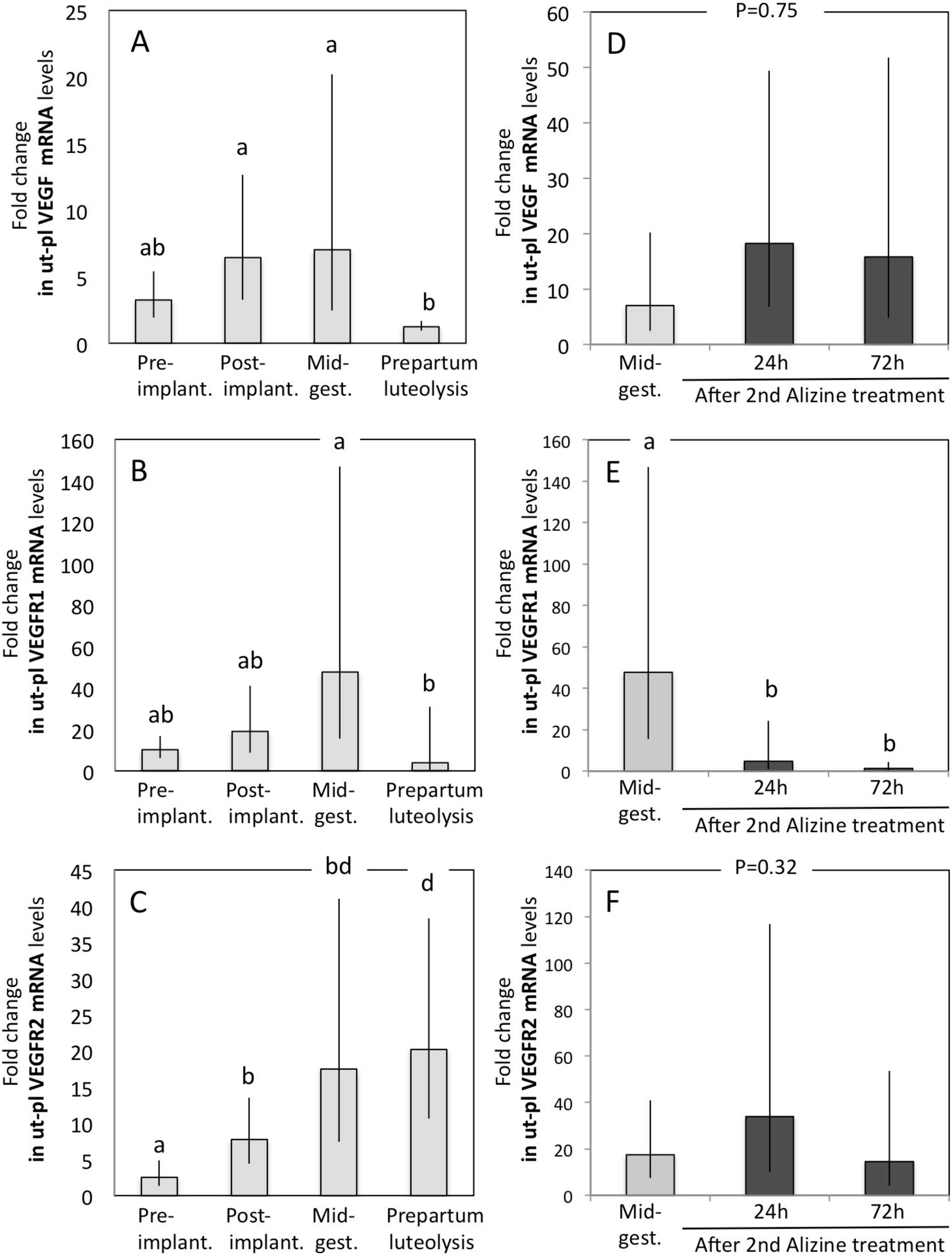
A
Fold change in ut-pl VEGF mRNA levels
25
20
15
10
5
0
ab
a
a
b
Pre-implant.
Post-implant.
Mid-gest.
Prepartum luteolysis
D
P=0.75
60
50
40
30
20
10
0
Mid-gest.
24h
72h
After 2nd Alizine treatment
B
Fold change in ut-pl VEGFR1 mRNA levels
160
140
120
100
80
60
40
20
0
a
ab
ab
b
E
b
b
C
Fold change in ut-pl VEGFR2 mRNA levels
45
40
35
30
25
20
15
10
5
0
bd
d
a
b
F
P=0.32
140
120
100
80
60
40
20
0
a vs b p<0.01
a vs bd p<0.001
a vs d p<0.001
b vs d p<0.05

Figure 2

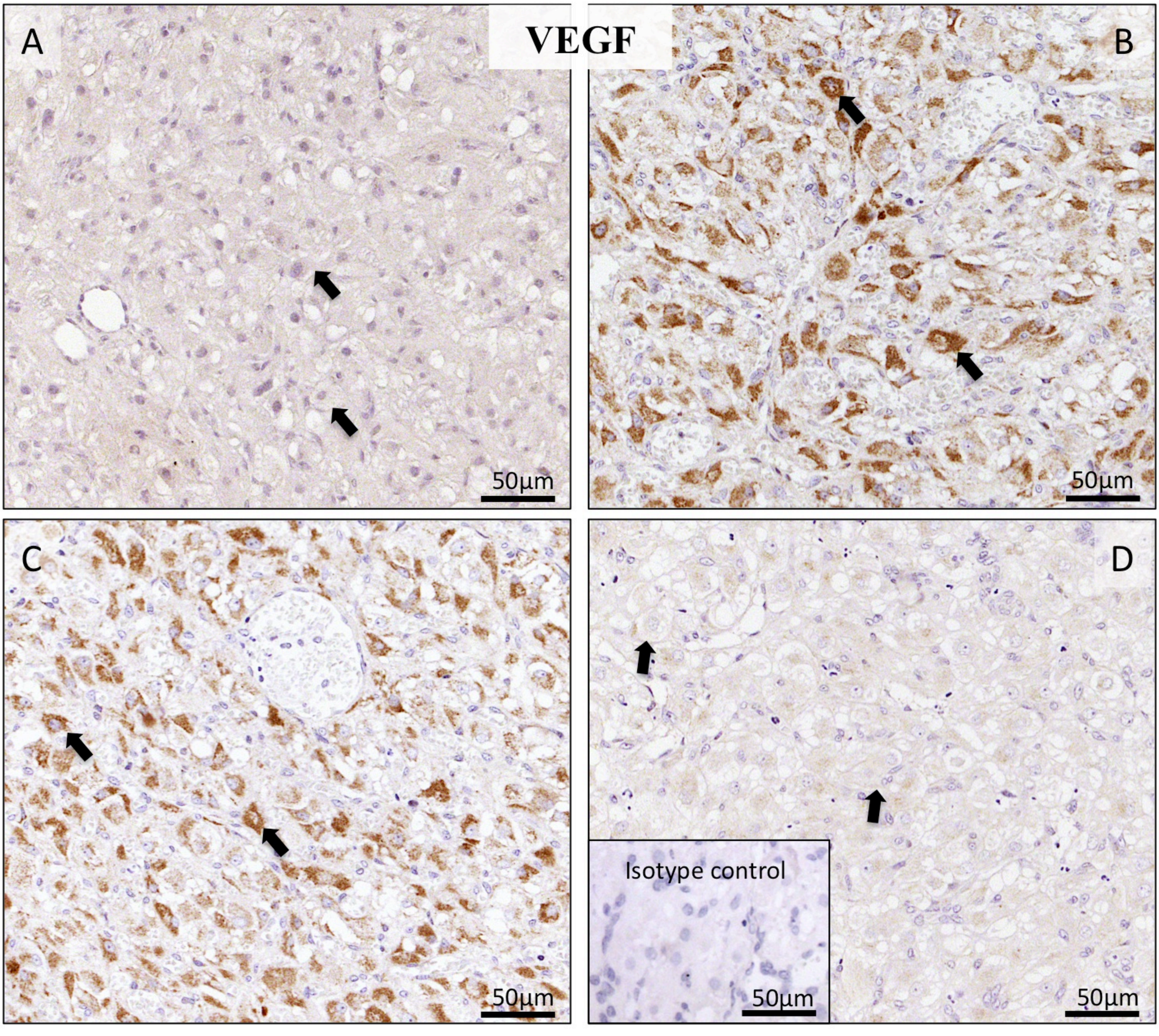

Figure 3

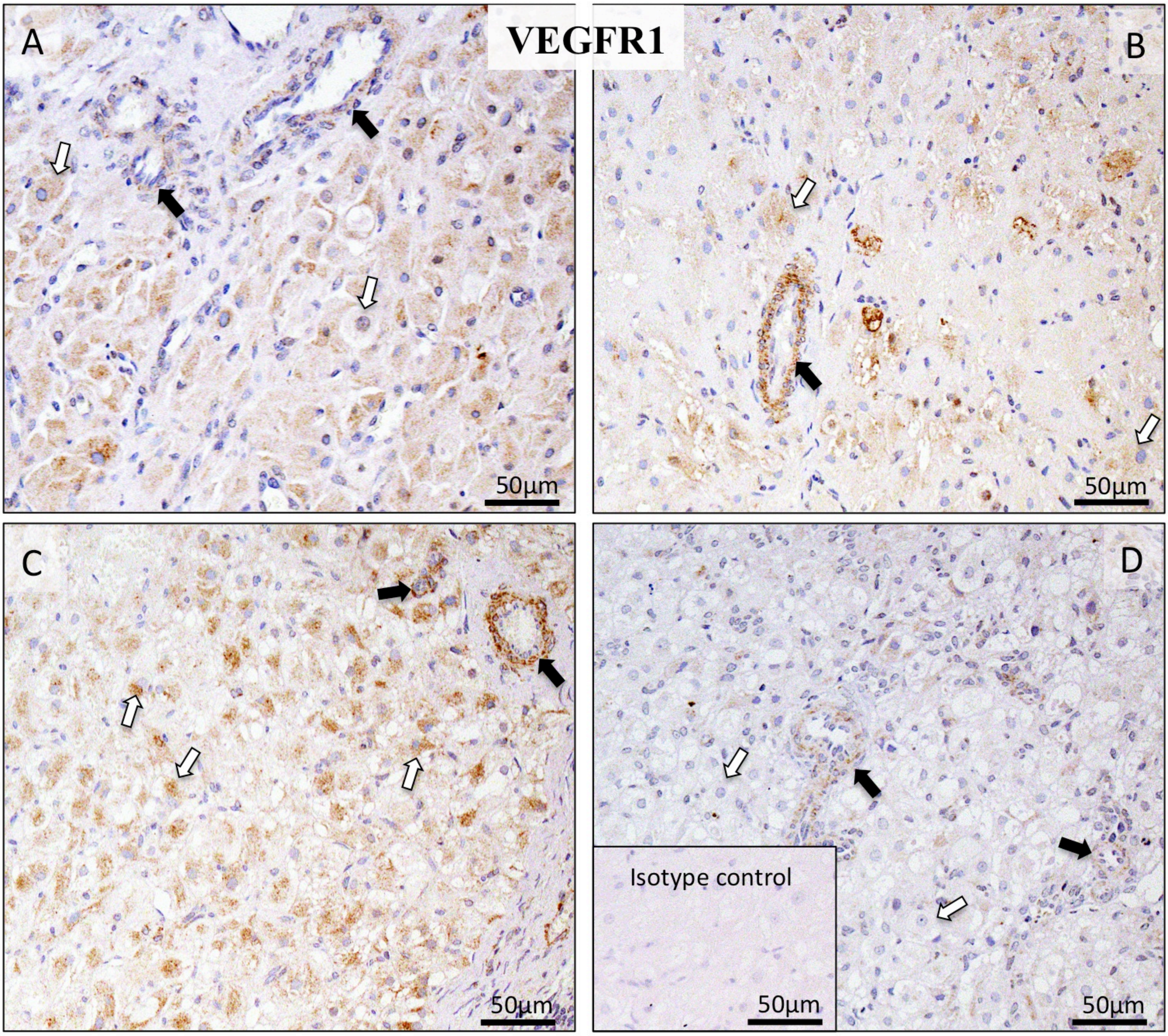

Figure 4

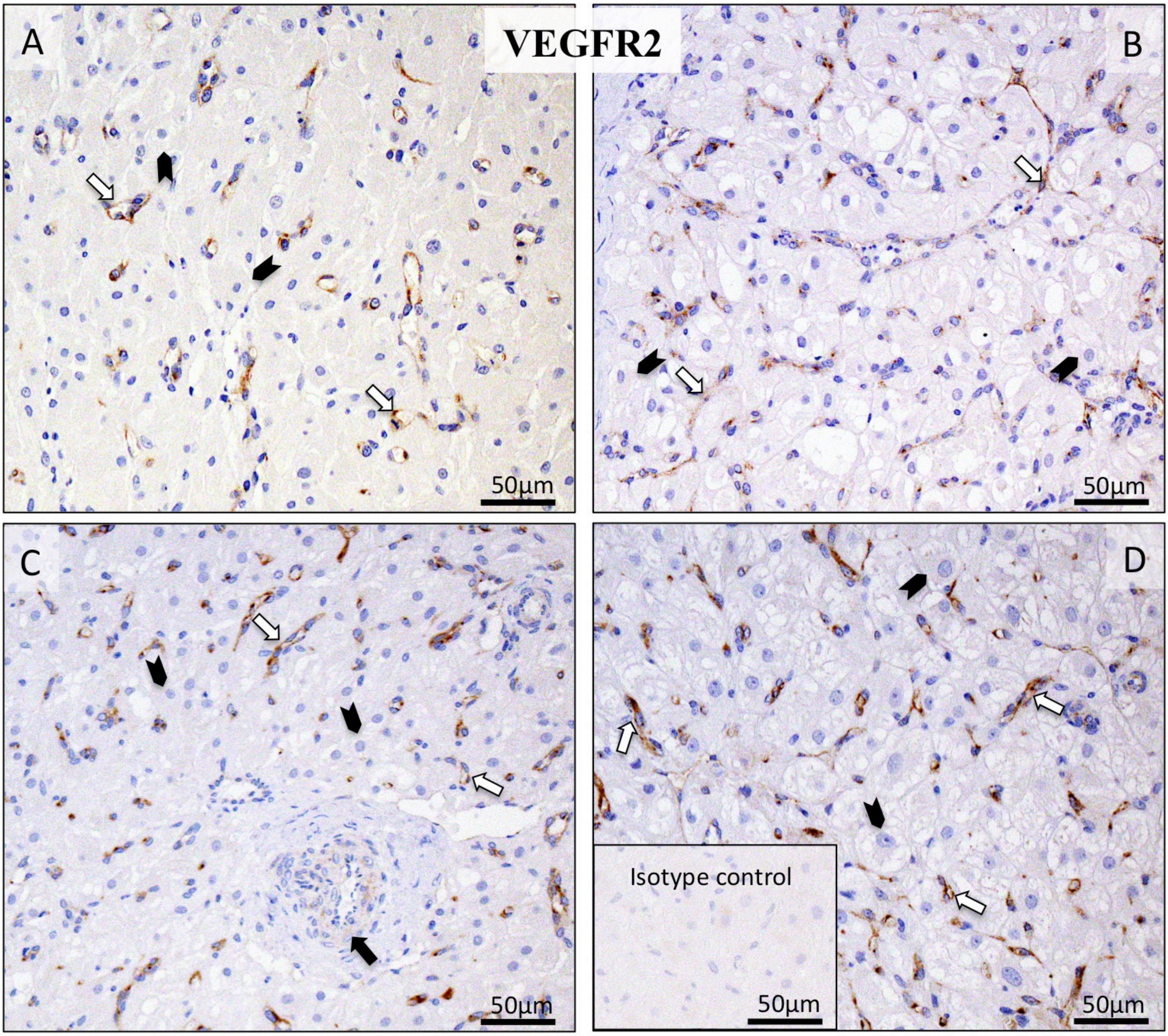

Figure 5

VEGF

A

50μm

B

50μm

C

50μm

D

50μm

E

25μm

F

50μm

G

50μm

H

Isotype control

25μm

25μm

Figure 6

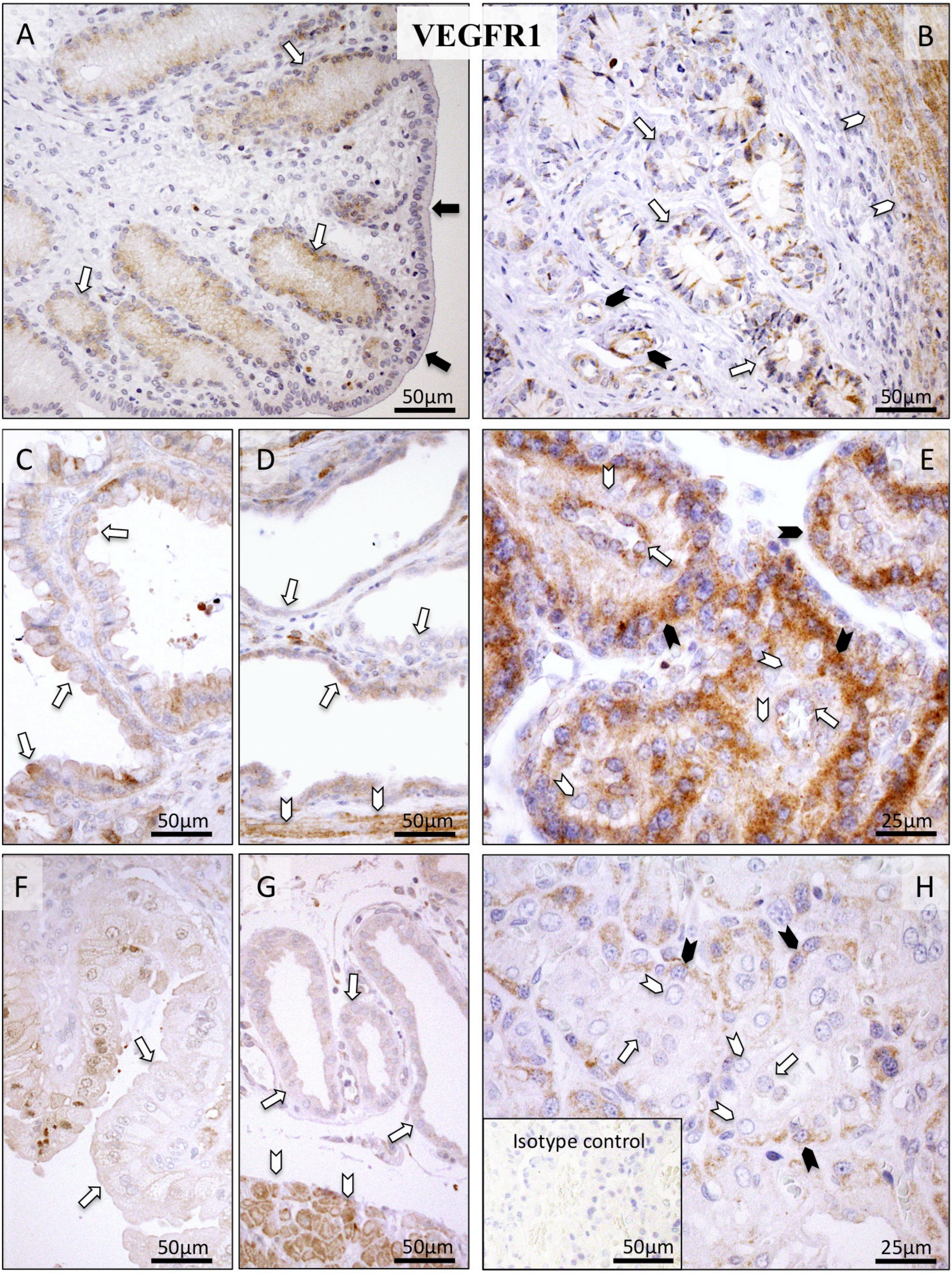
VEGFR1
A
50μm
B
50μm
C
50μm
D
50μm
E
25μm
F
50μm
G
50μm
H
Isotype control
50μm
25μm

Figure 7

VEGFR2

A

50µm

B

50µm

C

50µm

D

50µm

E

25µm

F

50µm

G

50µm

H

Isotype control

50µm

25µm

Figure 8

Primer	Accession Numbers	Primer Sequences	Product Length
GAPDH	AB028142	Forward: 5’-GCT GCC AAA TAT GAC GAC ATC A-3’ Reverse: 5’-GTA GCC CAG GAT GCC TTT GAG-3’ TaqMan probe: 5’-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3’	75bp
18SrRNA	FJ797658	Forward: 5’-GTC GCT CGC TCC TCT CCT ACT-3’ Reverse: 5’-GGC TGA CCG GGT TGG TTT-3’ TaqMan probe: 5’-ACA TGC CGA CGG GCG CTG AC-3’	125bp
VEGFA	NM001003175	Forward: 5’-GTG CCC ACT GAG GAG TTC AAC-3’ Reverse: 5’-CCC TAT GTG CTG GCC TTG AT-3’ TaqMan probe: 5’-CAC CAT GCA GAT TAT GCG GAT CAA ACC-3’	72bp
VEGFR1	AF262963	Forward: 5’-TGC CTG AAA CAG TGA GAA AGG A-3’ Reverse: 5’-TGC AGA ACT GTT TGC CAT TCC-3’ TaqMan probe: 5’-AAA GGC TGA GCA TTA CTA AAT CTG CCT-3’	81bp
VEGFR2	DQ269018/ NM001048024	Forward: 5’-TGA CAT GGC CTC GGT CAT T-3’ Reverse: 5’-TGT TGG TCG CTA ACA GAA GCA-3’ TaqMan probe: 5’-CTA CGT TCA AGA TTA CAG GTC TCC ATT-3’	75bp

Table 1